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14. ABSTRACT
The goal of this project is to determine the role of ERK/MAP kinase phosphorylation of the RUNX2 transcription factor in the metastasis of prostate cancer cells. In the first budget year, we achieved the following:
a. Generation of lentivirus and adenovirus sectors expressing WT RUNX2 and S301A, S319A phosphorylation-deficient RUNX2. Isolation of stable PC3 and LnCaP cell lines expressing WT and mutant RUNX2.
b. Demonstration that phosphorylation-deficient RUNX2 has reduced ability to induce metastasis-associated genes in PCa cells.
c. Demonstration that phosphorylation-deficient RUNX2 has reduced ability to stimulate in vitro migration of PC-3 PCa cells.
d. Demonstration that phosphorylation-deficient RUNX2 has reduced ability to induce VEGF synthesis and stimulate in vitro angiogenic activity of HDMECs.
e. Demonstration that phosphorylation-deficient RUNX2 has reduced ability to stimulate in vitro invasion of PC-3 cells.
f. Demonstration that RUNX2-S-319 phosphorylation is dramatically elevated in prostate cancer cells versus benign prostate hyperplasia and that elevated levels of P-RUNX2 persist in more advanced primary tumors and metastases.
These results support our overall hypothesis that RUNX2 phosphorylation is a critical determinant of tumorogenicity and metastasis of prostate tumor cells.

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Epigenetic Control of Prostate Cancer Metastasis: Role of Runx2 Phosphorylation

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INTRODUCTION

Bone metastasis leading to the formation of mixed osteoblastic and osteolytic lesions is seen in ~80% of men with metastatic prostate cancer (PCa)[1]. Little is known concerning the cellular signals controlling the metastatic behavior of the primary tumor. The presence of the RUNX2 transcription factor in prostate cancer cells has been related to invasive/metastatic behavior [2-5]. This project is based on preliminary studies suggesting that metastatic behavior of prostate tumors requires activation of the ERK/MAP kinase pathway, which phosphorylates the RUNX2 transcription factor. Previous work from our laboratory had shown that S301, S319 phosphorylation of RUNX2 is critical for its transcriptional activity in bone[6], and we hypothesized that these sites are also important for RUNX2-dependent metastatic activity. During the first year of this grant, efforts were focused on determining if RUNX2 phosphorylation is related to metastasis-associated cell properties. In addition, preliminary studies were conducted with prostate cancer tissue microarrays to determine if there is a relationship between RUNX phosphorylation levels and tumor outcome. In year 2, we initiated studies related to RUNX2 phosphorylation and epigenic changes in metastasis-associated genes, expanded studies on the role of RUNX2 phosphorylation in stimulating in vitro cell migration and invasion using non-tumorigenic cells, began in vivo metastasis experiments and completed a large tumor tissue microarray study to confirm the association of P-RUNX2 with prostate cancer in human samples.

BODY

Project Tasks: Accomplishments over the past year are listed according to original tasks described in the Statement of Work.

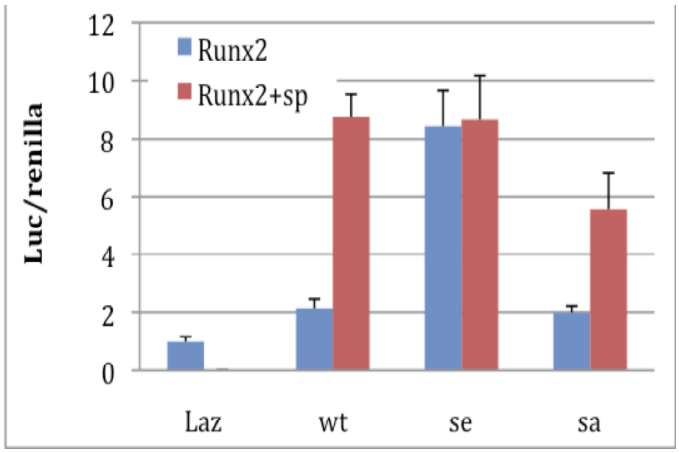
Task 1: Establish the relationship between MAP kinase signaling, Runx2 phosphorylation and transcriptional activity in normal prostate epithelial and PCa cells and determine how Runx2 phosphorylation controls VEGF gene expression.

a. Levels of total and P-ERK, total and P-Runx2 and Runx2-dependent activation of metastasis-associated genes will be compared in normal human prostate epithelial cells, K-ras-transformed prostate epithelial cells, non-metastatic LNCaP cells and three metastatic cell lines (PC-3, C4-2B, VPCa).

Comparison of cell lines showed that RUNX2 mRNA and protein levels were greatly elevated in the highly metastatic cell line, PC3, with progressively lower levels in C4-2B and LnCaP cells. Interestingly, the non-tumorigenic prostate cell lines, RWPE1/2 both contain relatively high levels of RUNX2. We are looking into the possibility that the P-RUNX2 levels in these cells are low, thus explaining why they have low migration/invasion activity. We are in the process of completing these studies.

b. Specific inhibitors of ERK/MAPK, Src and PI3K/AKT pathways as well as siRNA inhibition and overexpression of key pathway intermediates will be used to assess the relative importance of each signal transduction pathway in expression of metastatic genes.
We will do these studies in year 3.
c. Runx2-responsive regions of the proximal Vegf promoter will be identified using deletion/mutation analysis, functional assays and chromatin immunoprecipitation (ChIP).

Fig 1. Induction of MMP13 promoter activity by WT and S301A/S319A mutant Runx2. PC-3 cells were transfected with a 1.6 kb MMP13 promoter-luciferase reporter gene, renilla luciferase (for normalization of transfection) wild type



For these experiments, we decided to study the matrix metalloproteinase 13 (MMP13) promoter because it's regulation by RUNX2 has been more extensively studied relative to VEGF and is more directly related to the invasive behavior of cells (ref). Preliminary studies show that wild type Runx2, but not a phosphorylation-deficient S301A/S319A mutant can stimulate MMP13 gene expression and promoter activity. In contrast, an S301E/S319E mutant, where the charged amino acid (glutamate, E) mimicks phosphorylation had high activity even without MAP kinase activation (Fig. 1).

d. ChIP assays will be used to resolve whether binding of P-ERK to Vegf chromatin requires bound Runx2.

These studies are also being conducted with the MMP13 gene and will be completed in the coming year.

e. Use wild type Runx2, S301A/S319A (non-phosphorylated) or S301E/S310E (constitutively active) Runx2 mutants to determine whether Runx2 phosphorylation is necessary for histone phosphorylation, acetylation and activation of Vegf transcription.

Studies are being conducted with the MMP13 gene and will be completed in the coming year.

Task 2: Determine if Runx2 phosphorylation is necessary for *in vitro* and *in vivo* proliferative, invasive and metastatic behavior of PCa cells. stably transfected with wildtype Runx2 or Runx2 phosphorylation site mutants.

a. MLV-based retrovirus vectors will be developed that express β-galactosidase (negative control), wild type Runx2, S301A/S319A (non-phosphorylated) or S301E/S310E (constitutively active) Runx2 mutants. Although retroviruses were constructed to stably introduce wild type and mutant RUNX2 into cells, the low titer of these vectors and lack of selection markers to generate stable cells prompted us to develop a new set of viruses based on a lentivirus background. These vectors can be grown to high titer and transduce cells with high efficiency. Also a separate lentivirus will be used to introduce firefly luciferase into cells for use in cell tracking as part of in vivo metastasis experiments. We are in the process of constructing these vectors for use in generation of stable cell lines. The availability of lentivirus vectors will facilitate in vivo metastasis studies by allowing us to readily introduce wild type and mutant RUNX2 and the luciferase tracker into several prostate cell lines having high and low basal metastatic activity.

As reported last year, we also developed adenovirus vectors to express wild type and S301A/S319A mutant RUNX2. These vectors were used for analysis of metastasis-associated cellular activities in tissue culture (see below).

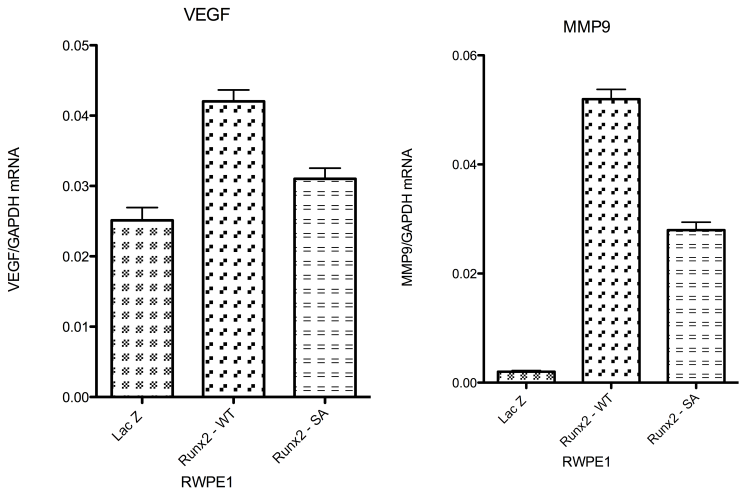
b. LnCaP cells with low intrinsic invasive/metastatic activity and Runx2 levels will be transfected with retrovirus vectors and stable lines will be isolated expressing β-Gal, wild type Runx2, S301A/S319A or S301E/S310E Runx2 mutants.

Initial attempts to develop stable lines of PC3 and LnCaP cells expressing β-galactosidase (negative control), wild type Runx2, S301A/S319A (non-phosphorylated) or S301E/S310E (constitutively active) Runx2 were not

successful. As described above, we are now pursuing the approach of using lentivirus vectors, which can be grown to higher titers and have higher transduction efficiencies. These vector properties will allow us to make stable luciferase-expressing RUNX2 lines of PCa cells having low (LnCaP) and high (PC3) basal metastatic activity as well as non-tumorigenic prostate cells (RWPE1 cells). These cells will then be used to measure the role of RUNX2 phosphorylation on in vivo metastasis.

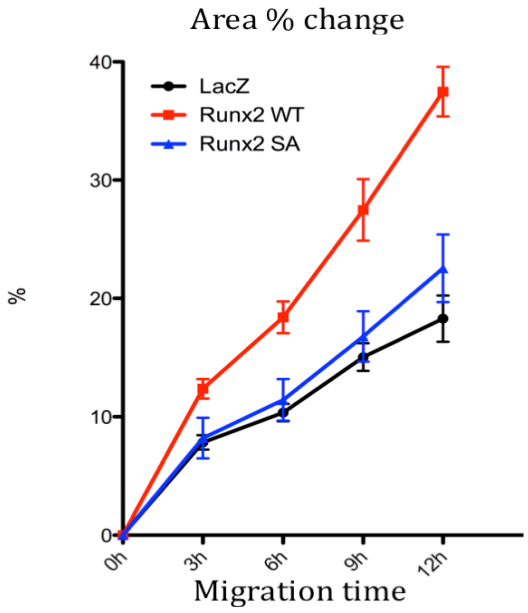
As an alternative approach to determine if RUNX2 phosphorylation is important for in vitro cell activity, adenovirus vectors encoding wild type and S310A/S319A RUNX2 were used to show that wild type, but not phosphorylation-deficient RUNX2 could stimulate metastasis-associated gene expression (MMP9, VEGF and osteopontin-OPN), angiogenesis, cell migration and invasion in vitro. Initial studies reported last year used PC3 cells for this analysis. Because these cells have fairly high basal metastatic activity, we recently repeated these studies using RWPE1 cells, an immortalized human prostate epithelial cell line that is not tumorigenic and has low in vitro migration and invasive activity. RWPE1 cells are a good model to determine if RUNX2 can stimulate a "normal" prostate cell to assume some of the properties of a metastatic cell.

Fig 2. Induction of Vegf and MMP9 mRNA with WT vs. phosphorylation-deficient RUNX2.



Induction of metastasis-associated gene expression in RWPE2 cells. WT RUNX2 was previously reported to induce several genes associated with tumor invasion/metastasis including osteopontin (Spp1), vascular endothelial growth factor (Vegf) and matrix metalloprotease 9 (MMP9). Transduction of RWPE1 cells with WT RUNX2 adenovirus strongly induced all these genes relative to an AdLacZ control virus while the RUNX2 S301A, S319A mutant had much less activity. Examples are shown for induction of VEGF and matrix metalloproteinase 9 (MMP9) mRNA, both of which are strongly induced by WT RUNX2, but only weakly induced by the S/A mutant (Fig 2).

Fig 3. WT but not phosphorylation-deficient RUNX2 stimulates migration of RWPE1 cells.

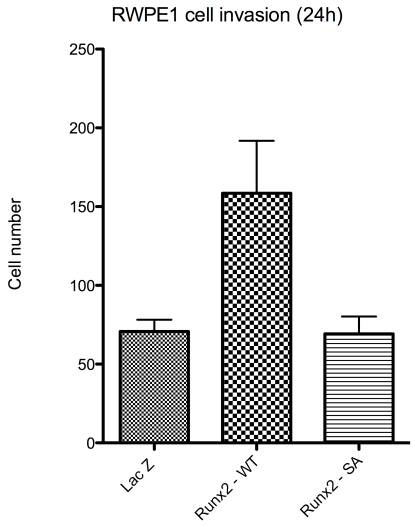


c. In vitro migratory activity of stable LNCaP lines will be compared. To measure cell migration, RWPE1 cells were transduced with AdLacZ, AdRUNX2wt or AdRUNX2 S301A/S319A mutant and plated onto glass slides. A central line of cells was removed using a Pasteur pipette and we measured the ability of cells to migrate into the cell-free area. As shown in Figure 3, WT RUNX2 clearly stimulates cell migration, while migration of cells transduced with the phosphorylation-deficient mutant RUNX2 is similar to that of control cultures.

d. The in vitro invasive activity of LNCaP lines will be compared using a matrigel invasion assay. To measure in vitro cell migration, RWPE1 cells transduced with AdLacZ control, AdRUNX2wt or AdRUNX2 S301A/S319A mutant were plated with serum-free medium in the upper well of a transwell apparatus containing a Millipore membrane precoated with Matrigel$_{TM}$. The lower chamber contained 10% fetal bovine serum which serves as a chemoattractant. Migration of cells through the Matrigel and Millipore membrane was scored by staining cells with Coomasie blue and counting (Fig. 4). AdRUNX2wt transduction of RWPE1 cells increased invasion through the Matrigel by approximately 2.3-fold while the AdRUNX2 S301A/S319A treated group was not significantly different from controls.

f. In vivo proliferative activity of LNCaP cell lines will be measured after subcutaneous and intrafemoral implantation into immunodeficient mice. The animal protocols for these studies were approved by the University of Michigan IACUC (Protocol PRO00003786) and USAMRMC Animal Care and Use Review Office (ACURO). This work will be pursued in year 3 of this project.

Fig 4. WT but not phosphorylation-deficient RUNX2 stimulates Matrigel$_{TM}$ invasion of RWPE1 cells.

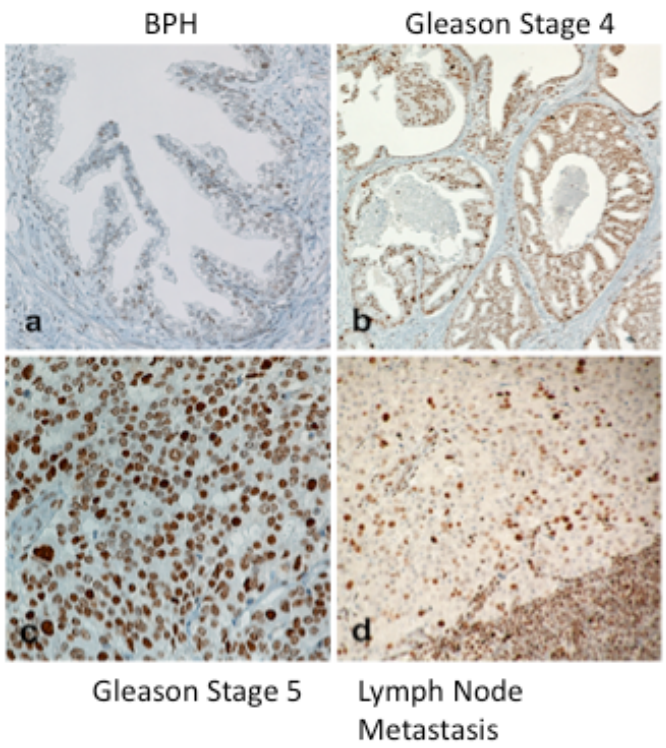


g. In vivo metastatic activity of LNCaP cell lines will be measured using an orthotopic (intraprostate implantation) model.
h. In vivo metastatic activity of LNCaP cell lines will be measured using an intracardiac injection model.
Both these subaims will be completed in year 3 of this project.

Task 3: Correlate phosphorylated (S319-P) Runx2, total Runx2 and P-ERK immunoreactivity with tumor outcome using a panel of human PCa tissue microarrays (TMAs) composed of normal prostate tissue, benign prostatic hyperplasia, prostatic intraepithelial neoplasia and prostate cancer.

a. TMAs provided by the Michigan Prostate Center SPORE will be immunostained using total and P-Runx2-specific antibodies as well as total and P-ERK antibodies.
b. Patterns of immunoreactivity will be correlated with tumor history; statistical models will be used to evaluate the predictive values and relationship to clinical parameters for each marker.

Fig. 5. Immunohistochemical staining of prostate cancer TMAs samples with anti-RUNX2-S319-P antibody. Shown are: a, benign prostate hyperplasia (BPH); b, Gleason stage 4 tumor; c, Gleason stage 5 tumor; d, lymph node metastasis.



We recently completed a comprehensive TMA analysis in collaboration with Dr. Guiseppe Panone (University of Foggia, Italy) who developed TMAs of prostate tissue that were stained with a P-RUNX2-specific antibody developed in the the project laboratory (antibody specifically recognizes RUNX2-S319-phosphate). TMAs included prostate diseases from 129 caucasian patients. Samples were distributed as follows: 106 tumor cases, represented by 93 primitive prostate cancers (PPC) treated by total prostatectomy with curative intent, and 13 lymph node metastases (LNM) from biopsy of inoperable primitive prostate adenocarcinomas; 15 cases of benign prostate hyperplasia (BPH); 8 cases of prostatitis (P) (4 p. granulomatosa and 4 p. xantogranulomatosa). This study is summarized in Table 1. Analysis revealed the following statistically-significant correlations between P-RUNX2 and prostate status:

1. Primitive prostate cancer, all sites, vs. benign prostate hyperplasia, $P < 0.0001$
2. Prostate cancer with high Gleason score vs. low Gleason score, $P < 0.05$
3. Prostate cancer with lymph node metastases vs. no lymph node metastases, $P < 0.05$
4. Primitive prostate cancer with prostatic intraepithelial neoplasia (PIN) vs. primitive prostate cancer without PIN. $P < 0.05$
5. Primitive prostate cancer versus prostatitis, $P < 0.0001$

Figure 5 compares P-RUNX2 staining intensity in representative TMA samples from this analysis and demonstrates the strong staining in all prostate cancer samples and near absence of staining in BPH.

In summary, these studies indicate that P-RUNX2 is an excellent biomarker for early and late stages of prostate cancer.

Table I. p-runx-2 immunohistochemical expression and statistical analyses on clinicopathological data from 129 patients.

GROUPS	*n°*	*MEAN p-Runx-2*	*Standard Deviation*	*Standard Error*	*Comparisons*	*ANOVA (P)*	*Student-Newman-Keuls P<0.05*
Low Gleason (LG-PPC)	68	53.65	29.20	3.54	*LG-PPC vs HG-PPC*	0.047	YES
High Gleason (HG-PPC)	25	67.92	33.01	6.60			
Perineural permeation (PNP-PPC)	75	58.43	30.39	3.51	*PNP-PPC vs NoNP-PPC*	0.549	NO
No neural permeation (NoNP-PPC)	18	53.56	32.79	7.73			
Vascular permeation (VP-PPC)	1	-	-	-	*VP-PPC vs NVP-PPC*	Impossible analysis	
No vascular permeation (NVP-PPC)	92	57.5	30.8	3.18			
PPC with lymph node metastasis at cTNM (N+PPC)	13	74.23	29.29	8.12	*N+PPC vs N0-PPC*	0.033	YES
PPC without lymph node metastasis at cTNM (N0-PPC)	80	54.76	30.29	3.39			
PIN-PPC	16	71.75	25.51	6.38	*PIN-PPC vs NoPIN-PPC*	0.041	YES
NoPIN-PPC	77	54.52	31.05	3.54			
Involved surgical margins (ISM-PPC)	56	58.36	30.81	4.12	*ISM-PPC vs FSM-PPC*	0.738	NO
Free surgical margins (FSM-PPC)	37	56.16	31.03	5.10			
Extra prostatic extension (EPE-PPC)	13	64.62	27.50	7.63	*EPS-PPC vs I-PPC*	0.370	NO
Intraprostatic (I-PPC)	80	56.33	31.25	3.49			
Primitive Prostate Cancer (PCC)	93	57.48	30.75	3.19	*PPC vs BPH*	0.000	YES
Benign prostatic hyperplasia (BPH)	15	1.87	2.07	0.53		0.000	YES
Primitive Prostate Cancer (PCC)	93	57.48	30.75	3.19	*PPC vs LNM*	0.464	NO
Lymph node metastases (LNM)	13	64.23	33.16	9.20			
Primitive Prostate Cancer (PCC)	93	57.48	30.75	3.19	*PPC vs P*	0.000	YES
Prostatitis (P)	8	4	0	0			

KEY RESEARCH ACCOMPLISHMENTS-YEAR 2

a. Generation of lentivirus and adenovirus vectors expressing WT RUNX2 and S301A, S319A phosphorylation-deficient RUNX2.

b. Demonstration that phosphorylation-deficient RUNX2 has reduced ability to induce metastasis-associated genes in RWPE1 non-transformed human prostate epithelial cells.

c. Demonstration that phosphorylation-deficient RUNX2 has reduced ability to stimulate in vitro migration of RWPE1 cells.

d. Demonstration that phosphorylation-deficient RUNX2 has reduced ability to stimulate in vitro invasion of RWPE1 cells.

e. Demonstration that RUNX2-S-319 phosphorylation is dramatically elevated in prostate cancer cells versus benign prostate hyperplasia or prostatitis and that elevated levels of P-RUNX2 persist in more advanced primary tumors and metastases.

REPORTABLE OUTCOMES

The following RUNX2 expression vectors were generated :
AdRUNX2 WT- expresses wildtype murine RUNX2 at high levels in prostate cancer cells.
AdRUNX2 Mut- expresses S301A, S319A phosphorylation-deficient RUNX2 at high levels in PCa cells.
Lenti-RUNX2 WT- expresses wildtype murine RUNX2 at high levels in prostate cancer cells.
Lenti-RUNX2 Mut- expresses S301A, S319A phosphorylation-deficient RUNX2 at high levels in PCa cells.

Results from this project have been reported in abstract form and presented at the 11th International Conference of Cancer-Induced Bone Disease, Chicago, IL, November 30-December 3, 2011.

Zhao Z, Ge C, Pannone G, Bufo P, Santoro A, Sanguedolce F, Tororella S, Mattoni M, Papagerakis P, Papagerakis S and **Franceschi RT**. RUNX2 phosphorylation as a prognostic marker of metastatic disease in prostate cancer. 11th International Conference of Cancer-Induced Bone Disease Proceedings (2011) Abst P145.

We are currently preparing a full manuscript describing this work that will be submitted to either Cancer Research or Oncogene.

CONCLUSIONS

We made good progress for all 3 tasks in this project. Specifically, we clearly showed that the RUNX2 transcription factor is able to induce the expression of metastasis-associated genes and increase in vitro migratory, angiogenic and invasive properties of prostate cancer cell lines. Furthermore, RUNX2 must be phosphorylated to stimulate these activities. A tissue microarray study with a total of 129 patient samples showed a good correlation between the presence of RUNX2-S319-P in prostate tissue and neoplastic activity with minimal staining observed in benign prostate hyperplasia or prostatitis. Efforts in the coming year will be focused on examining the importance of RUNX2 phosphorylation in tumor growth and metastasis in vivo as well as to examine the mechanism through which RUNX2 phosphorylation controls the activity of metastasis-related genes.

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